Current Biology 19, 330–334, February 24, 2009 ©2009 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2009.01.018

Report

CO_2 Regulates White-to-Opaque Switching in *Candida albicans*

Guanghua Huang,[1] Thyagarajan Srikantha,[1] Nidhi Sahni,[1] Song Yi,[1] and David R. Soll[1,*]
[1]Department of Biology
The University of Iowa
Iowa City, IA 52242
USA

Summary

To mate, *Candida albicans* must undergo homozygosis at the mating type-like locus *MTL* [1, 2], then switch from the white to opaque phenotype [3, 4]. Paradoxically, when opaque cells are transferred in vitro to 37°C, the temperature of their animal host, they switch en masse to white [5–7], suggesting that their major niche might not be conducive to mating. It has been suggested that pheromones secreted by opaque cells of opposite mating type [8] or the hypoxic condition of host niches [9, 10] stabilize opaque cells. There is, however, an additional possibility, namely that CO_2, which achieves levels in the host 100 times higher than in air [11–13], stabilizes the opaque phenotype. CO_2 has been demonstrated to regulate the bud-hypha transition in *C. albicans* [14, 15], expression of virulence genes in bacteria [16], and mating events in *Cryptococcus neoformans* [14, 17]. We tested the possibility that CO_2 stabilizes the opaque phenotype, and found that physiological levels of CO_2 induce white-to-opaque switching and stabilize the opaque phenotype at 37°C. It exerts this control equally under anaerobic and aerobic conditions. These results suggest that the high levels of CO_2 in the host induce and stabilize the opaque phenotype, thus facilitating mating.

Results and Discussion

CO_2 Stimulates White-to-Opaque Switching

The CO_2 content of air is 0.03%, but in the animal host it ranges between 4.5% and 30% [11–13]. When white cells of five natural strains homozygous at the *MTL* locus (see Table S1, available online) were plated and incubated in air containing 5% CO_2 at 25°C, the frequency of switching, measured as the proportion of total colonies that are white with opaque regions or completely opaque, was 4-fold to 16-fold higher than in air (Figures 1A and 1B). When plated and incubated in air containing 20% CO_2, the frequency was 10-fold to 105-fold higher than in air. CO_2 also induced white-to-opaque switching at 37°C (Figure S1). Variability existed among strains both in air and in the test concentrations of CO_2, but switching was induced by high concentrations of CO_2 in all strains (Figure 1B).

CO_2 Blocks Opaque to White Switching

When opaque cells of test strains were plated and incubated at 25°C in air, two (WO-1, GH1012) exhibited moderate frequencies of switching, and three (P37005, P97099, and P78048) exhibited high frequencies of switching (Figure 2A). In air containing 5% CO_2, the frequency of switching by opaque cells of all five strains was reduced to low or negligible levels (Figure 2A). In addition, 5% CO_2 blocked temperature-induced mass conversion of opaque to white [5–7] after a temperature shift from 25° to 37°C (Figure 2B). At 37°C in air, both white and opaque cells multiplied, but whereas white cells maintained their phenotype, opaque cells converted en masse to white (Figure 2C). In 5% CO_2, however, both white and opaque cells multiplied and maintained their respective phenotypes (Figure 2C).

Phase-Specific Gene Expression

To test whether CO_2-induced white-to-opaque switching was accompanied by associated changes in gene expression, two white-specific genes, *WH11* [18] and *EFG1* [19, 20], and two opaque-specific genes, *OP4* [21] and *WOR1* (*TOS9*) [22–24], were analyzed by northern blot hybridization. In air, the transcript levels of the two white-specific genes remained high, and the transcript levels of the two opaque-specific genes remained negligible (Figure 3). In 5% or 20% CO_2, however, the transcript levels of the two white-specific genes decreased to negligible levels, whereas those of the two opaque-specific genes increased dramatically (Figure 3).

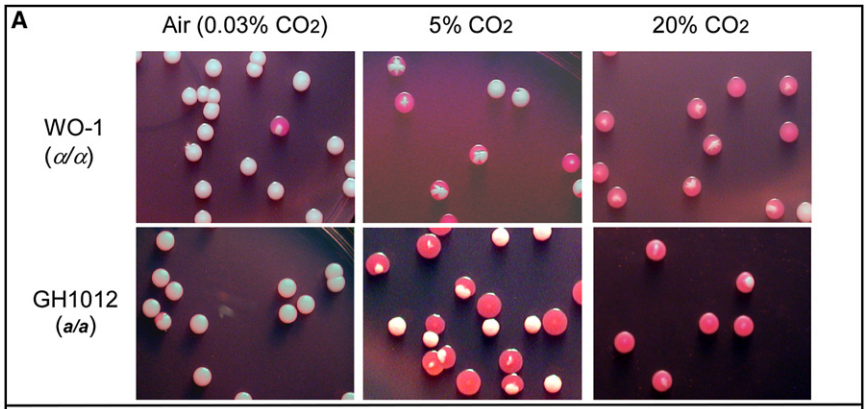
CO_2 Facilitates Mating

Because CO_2 induced white-to-opaque switching and stabilized the opaque phenotype, we predicted it would facilitate mating in white populations. To test this, crosses were performed in air and 5% CO_2, by mixing white cells of strain WUM5A (α/α, *ura3*$^-$) and MMY278 (***a***$/\alpha\Delta$, *ade2*$^-$), and white cells of strains CHY477 (***a***Δ/α, *ade2*$^-$) and 3UM5A (***a/a***, *ura3*$^-$) (Table S1). Five percent CO_2 increased the mating efficiency in the two crosses by 583-fold and 395-fold, respectively (Table S3).

Roles of Carbonic Anhydrase, Adenylate Cyclase, and Ras1

At low atmospheric CO_2 levels, carbonic anhydrase (CA) catalyzes the interconversion of CO_2 and carbonic acid (HCO_3^-), which is necessary for obtaining CO_2 effects, presumably through HCO_3^- signaling; at high atmospheric CO_2 levels, diffusion across the plasma membrane is high enough so that the uncatalyzed formation of HCO_3^- is sufficient [15]. In *C. albicans*, deletion of the gene for carbonic anhydrase, *NCE103*, blocks growth in air (0.03% CO_2), but not in air supplemented with 5% CO_2 (Figure 4A), and affects hypha induction at low but not high CO_2 concentrations [15]. We tested whether carbonic anhydrase plays a similar role in the induction of switching by CO_2 by analyzing the null mutant *nce103*Δ (Table S1). Because *nce103*Δ cells did not grow in air [15], white cells of the mutant were first grown on agar in air containing 1% CO_2, then plated on fresh agar plates and incubated in 1% or 5% CO_2 to test for stimulation. Although more than 90% of the colonies formed in 1% CO_2 (low CO_2) by white cells of wild-type and complemented strains contained opaque regions, only 22% and 19% of the colonies formed in 1% CO_2 by white cells of strain *nce103*Δ contained opaque

*Correspondence: david-soll@uiowa.edu



B Quantitation

		Air (0.03% CO_2)		5% CO_2		20% CO_2	
Strain	*MTL* genotype	Total colonies	%Op colonies or wh colonies with op regions	Total colonies	%Op colonies or wh colonies with op regions	Total colonies	%Op colonies or wh colonies with op regions
WO-1	α/α	1379	5.4 ± 1.6	1101	88.8 ± 1.9 (16 x)	957	93.5 ± 3.0 (17 x)
GH1012	a/a	1300	9.1 ± 1.6	989	84.9 ± 2.2 (9 x)	720	90.6 ± 2.7 (10 x)
P37005	a/a	2677	0.9 ± 0.4	1811	6.6 ± 1.2 (7 x)	772	53.4 ± 3.0 (59 x)
P97099	α/α	1340	0.9 ± 0.5	1286	6.1 ± 0.7 (7 x)	690	94.5 ± 3.5 (105 x)
P78048	α/α	1170	0.6 ± 0.2	950	2.3 ± 0.3 (4 x)	690	20.3 ± 2.5 (34 x)

Figure 1. High Concentrations of CO_2 Induce Switching from the White, Wh, to Opaque, Op, Phenotype in *C. albicans*

White cells of either α/α or **a/a** strains were plated on agar and then incubated in air, which contained 0.03% CO_2, air (95%) containing 5% CO_2, or air (80%) containing 20% CO_2 (Supplemental Experimental Procedures). The agar contained phloxine B, which stained opaque colonies or opaque regions of white colonies red [28].
(A) Representative fields of colonies for one α/α (WO-1, FC4) and one **a/a** strain (GH1012).
(B) Quantitation of the frequency of switching measured as the proportion (%) of total colonies that were white with opaque regions or predominately opaque. The total number of colonies is the sum of three experiments. The "% Op colonies or Wh colonies with Op regions" represents the mean for the three experiments ± standard deviation. The fold difference with air is presented for the means at 5% and 20% CO_2. The p values calculated by the Student's two-tailed t test for switching frequencies in 5% and 20% CO_2 compared with those in air were all less than 0.05.

regions (Figure 4B). At 5% CO_2 (high CO_2), however, over 95% of the colonies formed by white cells of both control and mutant strains contained opaque regions (Figure 4B). Therefore, carbonic anhydrase was necessary for wild-type levels of switching at low (1%) CO_2, but it was not necessary for maximum levels at high (5%) CO_2, which is similar to the effects described for CO_2-induced filamentation [15]. Klengel et al. [15] demonstrated that CO_2 induced the bud-hypha transition by stimulating adenylyl cyclase. This cAMP-dependent pathway in turn required Ras1 [25]. We therefore tested the CO_2 effect on switching in null mutants of the adenylyl cyclase gene (*cdc35Δ*) and *RAS1* (*ras1Δ*) (Table S1). In both mutants, the frequency of switching was lower than that of the control strain in air (Figure 4C). In 1% CO_2, switching increased in the two mutant strains, but to levels far below those of control cells (Figure 4C). At 20% CO_2, however, switching was maximal, as in control cells (Figure 4C). Therefore, both adenylyl cyclase and Ras1, like carbonic anhydrase, enhanced switching in air and enhanced induction by low but not high concentrations of CO_2.

WOR1 and *CZF1*

Because the master switch locus *WOR1* (*TOS9*) regulates the white-to-opaque transition [22–24], and overexpression of the transcription factor Czf1 promotes that switch [26], we analyzed the induction of switching by CO_2 in the mutants *wor1Δ* and *czf1Δ* (Table S1). As expected, switching did not occur in air or at 20% CO_2 in white cells of *wor1Δ* (Figure 4C). CO_2 did, however, induce switching in white cells of *czf1Δ*, but the induced frequencies in 1% and 20% CO_2 were lower than that of control cells, as was the basal frequency in air (Figure 4C). As expected, CO_2 did not induce switching in **a**/α cells (Figure 4C).

CO_2 and O_2

Dumitru et al. [9] found that under anaerobic conditions at 37°C, opaque cells switched to white at a lower frequency than under aerobic conditions, suggesting that hypoxia stabilized the opaque phenotype. However, opaque cells also divided at a slower rate under anaerobic conditions [9], and switching requires cell division [7, 18]. Therefore, the observed reduction in frequency could be due to the decrease in growth rate. Ramirez-Zavala et al. [10] demonstrated that anaerobic conditions induced white cells to switch to opaque, and that induction was regulated by Czf1. Their method to remove O_2, however, generated 18% CO_2 in the culture jars, a concentration we found induces a maximal rate of switching (Figure 1) and a maximum level of phenotypic stabilization (Figure 2A). To address whether hypoxia alone can stimulate switching, white cells were plated and incubated in either N_2 (99.97%) containing no O_2 and 0.03% CO_2 (the CO_2 concentration in air), or N_2 (99.47%) containing 0.5% O_2 (a 40-fold reduction from that in air) and 0.03% CO_2. Rather than stimulating switching, the frequency was reduced 8-fold and 12-fold, respectively, from that in air (Table S4). When white cells were plated and incubated in either N_2 (90%) containing no O_2 and 10% CO_2, or N_2 (89.75%) containing 0.5% O_2 and 9.75% CO_2, the frequencies of switching were 98.9% and 97.9%, respectively (Table S4). These results demonstrate that hypoxia does not induce white-to-opaque switching and that CO_2 induces switching equally in the absence and presence of O_2.

Conclusions

We set out to resolve the paradox that in vitro, the mating-competent opaque cell phenotype was unstable at 37°C [5–7, 18], but the body temperature of the animal host, the major environment niche of *C. albicans*, was 37°C, suggesting that the host was not conducive to opaque cell mating. We presented evidence that exposure of white cells to the high levels of CO_2 found in host niches induced switching from white to opaque, then maintained the opaque phenotype, thus providing an explanation for this paradox. CO_2 induced white-to-opaque switching equally well under anaerobic and aerobic conditions, the former also a characteristic of most host niches. Our results indicated that hypoxia was not an inducer of white-to-opaque switching. At low, but not high,

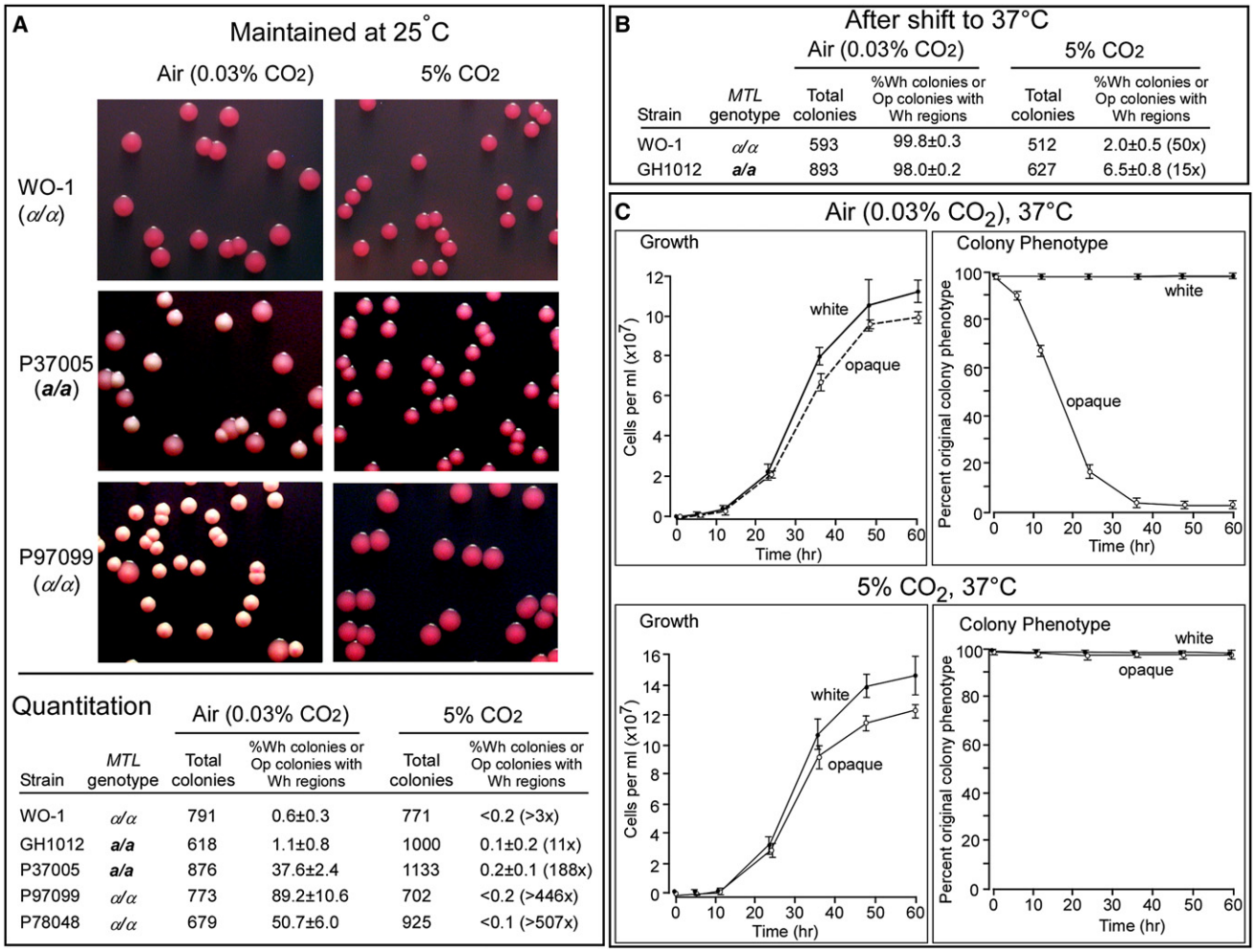


Figure 2. CO_2 Stabilizes the Opaque Phenotype at 25° and 37°C and Does Not Interfere with Cell Multiplication

(A) Opaque cells of ***a/a*** and α/α cells were plated on agar and monitored for switching according to the general procedure outlined in the legend to Figure 1 and Supplemental Experimental Procedures. The p values calculated by the Student's two-tailed t test of the switching frequencies of P37005, P97099, and P78048 in 5% CO_2 compared with those in air were less than 0.05. The data are the mean and standard deviation of three independent experiments.

(B) Opaque cells were plated at 37°C and analyzed for switching to white, according to methods outlined in Supplemental Experimental Procedures. The p values calculated by the Student's two-tailed t test of WO-1 and GH1012 in 5% CO_2 compared with those in air were less than 0.05. The data are the mean and standard deviation of three independent experiments.

(C) White or opaque cells were grown in liquid cultures at 37°C in air or air containing 5% CO_2, and monitored for cell number and cell phenotype (Supplemental Experimental Procedures). Cell phenotype was monitored by plating aliquots at time intervals and counting the proportion of colonies with regions of alternative phenotype. The data points and error bars represent the mean and standard deviation of three experiments.

concentrations of CO_2, induction was selectively enhanced by carbonic anhydrase, adenylate cyclase, and Ras1. Similar dependencies have been observed for CO_2 induction of hypha formation [15], which might not be surprising given other similarities between the white-opaque transition and the bud-hypha transition [27]. At both low and high CO_2 concentrations, the transcription factor Czf1 promoted the induction of switching. Czf1 had previously been shown to promote white-to-opaque switching [10, 26]. The observation that the basal rates of switching in air and the induced rates in low CO_2 were depressed in the mutants *nce103Δ*, *cdc35Δ*, and *ras1Δ* suggested that what has been referred to as "spontaneous" switching from white to opaque in air [5] might in fact represent induced switching by the low level of CO_2 (0.03%). We therefore propose that in the host, it is the high level of CO_2 that induces the white-to-opaque switch, then stabilizes the mating-competent opaque cell, thus facilitating mating.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, one figure, and four tables and can be found with this article online at http://www.current-biology.com/supplemental/S0960-9822(09)00557-0.

Acknowledgments

The authors are indebted to Joachim Morschhäuser at the University of Würzburg, Germany, Alexander Johnson at the University of California, San Francisco, and Gerald R. Fink at Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology for the generous gifts of plasmids and strains. The authors are also indebted to Karla Daniels for help in assembling the figures and Claude Pujol for comments in the

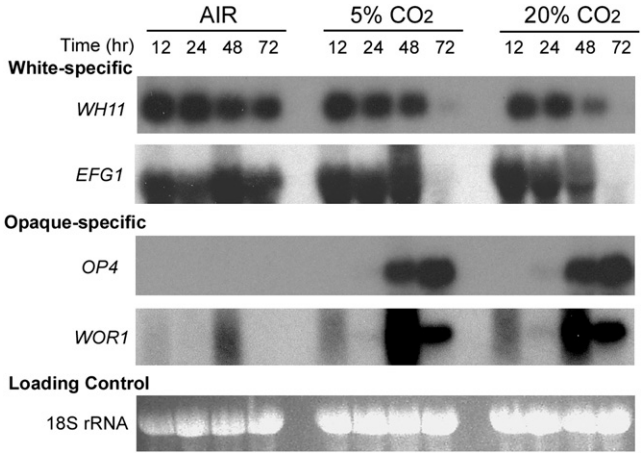


Figure 3. CO_2-Induced Switching from White to Opaque Is Accompanied by Downregulation of White-Specific and Upregulation of Opaque-Specific Genes

Northern blot hybridization was performed for white- and opaque-specific genes in white cell cultures of strain WO-1 for 0.5, 1, 2, and 3 days on agar in air, air containing 5% CO_2, or air containing 20% CO_2. The ethidium-bromide-stained 18S ribosomal RNA bands are shown to demonstrate equal loading of lanes.

manuscript. This research was supported by NIH grant AI2392 and the Developmental Studies Hybridoma Bank-Microbe at Iowa.

Received: October 2, 2008
Revised: December 22, 2008
Accepted: January 7, 2009
Published online: February 5, 2009

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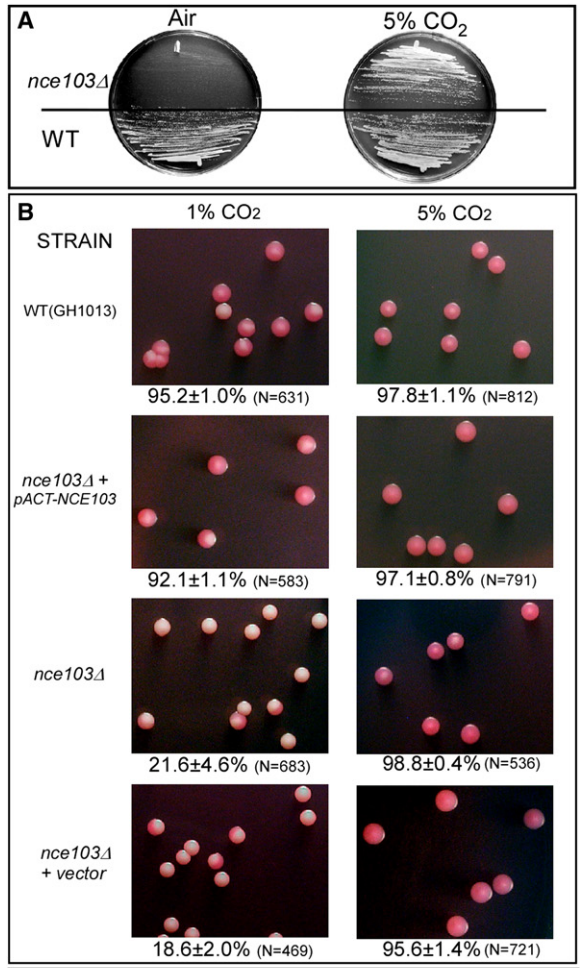

C

Strain	% op colonies or colonies with op regions: Air (0.03% CO_2)	1% CO_2	20% CO_2
WT(GH1013)	1.7 ± 0.7	21.9 ± 4.6	97.2 ± 1.0
ras1Δ	0.8 ± 0.7	3.4 ± 0.5	90.4 ± 3.4
cdc35Δ	0.03 ± 0.05	0.3 ± 0.3	97.6 ± 0.8
WO-1(Soll1)	4.4 ± 1.1	19.5 ± 5.3	97.2 ± 1.3
czf1Δ	0.1 ± 0.1	3.3 ± 0.8	34.0 ± 3.0
wor1Δ	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
SC5314(***a***/α)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
5314**a** (***a***/***a***)	0.4 ± 0.6	16.3 ± 3.4	98.2 ± 1.7
5314α(α/α)	0.6 ± 0.5	9.8 ± 2.9	99.4 ± 1.1

Figure 4. CO_2 Induction of Switching from White to Opaque in Null Mutants of Carbonic Anhydrase, Adenylate Cyclase, Ras1, the Master Switch Locus *WOR1*, and the Transcription Regulator Czf1

The representative mutants were *nce103Δ*, *cdc35Δ*, *ras1Δ*, *wor1Δ*, and *czf1Δ*.

(A) Agar cultures demonstrating that the *nce103Δ* mutant does not grow in air, but does grow in 5% CO_2.

(B) Representative fields of colonies formed by white cells of parent strain GH1013 (Table S1) and *nce103Δ* in air containing 1% or 5% CO_2. Fields of control strains *nce103Δ+pACT-NCE103* (the complemented *nce103Δ* mutant) and *nce103Δ*+vector (*nce103Δ* transformed only with the vector) are also presented.

(C) The switching frequencies of the tested mutants, wild-type controls, and an ***a***/α control, monitored according to methods outlined in the legend to Figure 1 and Supplemental Experimental Procedures. The data are the mean and standard deviation of three independent experiments.

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